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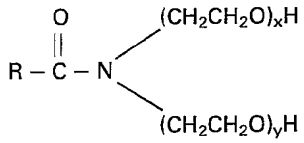
(54) **Reagent for reducing turbidity in a biological fluid**

(57) Biological fluids such as serum or plasma are often turbid which makes their analysis difficult. Reagents for reducing this turbidity comprise a surfactant of the formula



where R is alkyl or alkenyl of 5 to 17 carbon atoms, and x and y are integers whose sum is no more than 11; and cholesterol esterase or lipase. The reagents are used as buffered aqueous solutions.

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SPECIFICATION

Reagent for reducing turbidity in a biological fluid

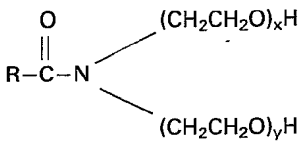
This invention relates generally to a method for reducing turbidity in a biological fluid sample such as human serum and to a reagent for that purpose.

Turbidity in a biological fluid sample can cause severe problems in the assay of that sample. it can result in poor or incorrect assay readings and therefore highly questionable determinations. Thus, turbidity in serum and plasma samples is usually a serious problem in clinical photometric analysis. It gives false data and often yields misleading photometric determinations of serum ingredients. The turbidity is believed to be caused primarily by the elevation of triglycerides in serum of patients having hyperlipoproteinemia with or without elevated total cholesterol. Abnormal elevation of cholesterol in serum has been shown to correlate with a high risk of artherosclerosis. Determination of cholesterol and triglycerides is important since accurate data will help the doctor to diagnose patients with hyperlipoproteinemia and to predict certain heart disease. Other tests such as aspartate aminotransferase (GOT), alanine aminotransferase (GPT) and lactate dehydrogenase (LDH), etc. Have also suffered from the same difficulities as the cholesterol or triglyceride determinations, as mentioned above, when turbid samples are examined.

Clinical tests of turbid sera have been handled in the past by treating samples with a high concentration of surfactants such as polyoxyethylated lauric acid (U.S. Patent Nos. 3,853,465 and 4,184,848). Since only surfactants were used, a rather high concentration of surfactant was needed for an effective clearing. However, high concentrations of surfactants sometimes interfere with other chemical or enzyme reactions and can so cause complications in the analysis.

We have now devised a reagent useful for reducing turbidity in biological fluid samples, in which a surfactant is used (preferably in relatively low concentration) together with an enzyme (cholesterol esterase or lipase).

According to the invention, there is provided a reagent for reducing turbidity in a biological fluid sample, which reagent comprises at least one surfactant of the formula:



wherein R is alkyl or alkenyl containing from 5 to 17 carbon atoms; x and y are integers whose sum is not greater than 11; and an enzyme selected from cholesterol esterase or lipase or mixtures thereof.

The invention also includes a method of assay of a biological fluid sample which includes the step of reducing turbidity in the sample by mixing it with a reagent of the invention.

The exact mechanism by which the reagents of the invention work is not yet known. It is possible to speculate, however, that in cases where patients have hyperlipemia, the turbidity found in serum samples is due primarily to an elevated triglyceride content. Triglycerides are water-insoluble, and usually buried inside the fat core with cholesterol esters in lipoprotein complex. The clearing of a lipemic sample must be brought about by the disruption of lipoprotein by a surfactant such as lauric acid diethanolamide (DEA), followed by the hydrolysis of triglycerides by the enzyme base. The surfactant also aids the dissolution of fatty acids released therein. Without enzyme, there is no effective clearing.

A preferred reagent of the invention is one in which x and y are each 1. The reagents are preferably in aqueous buffered form, and, when the enzyme is cholesterol esterase preferably comprise from about 0.05 g/dl to about 2.5 g/dl of surfactant, more preferably from 0.1 g/dl to about 0.5 g/dl, and at least about 0.025 U/ml of cholesterol esterase, based on the total formulation. The resulting aqueous buffered reagent will have a pH of from about 5.5 to about 7.0.

When the reagent contains a lipase enzyme, the surfactant preferably comprises from about 0.05 g/dl to about 2.5 g/dl, more preferably from 0.1 g/dl to about 0.5 g/dl, and the enzyme comprises at least about 1.0 U/ml of the resulting formulation, the pH being from about 5.5 to about 8.0.

In both enzyme reagents, the buffer employed can, for example, be a maleate, in the form of the sodium or potassium salt; a phosphate; a borate; a citrate; a succinate; and imidazole-acetate buffer; tris; etc. Any suitable buffer can be used, i.e. any buffer which will maintain a substantially constant pH in the desired range without interfering with any of the components.

When a maleate buffer is used, e.g. the potassium or sodium salt, it is generally added in amounts to provide from about 0.05 M to about 0.5 M and the pH of the resulting reagent is from about 5.0 to about 7.0. Similar concentrations are used for the other buffers.

In addition to the above components, a solubility enhancer can be included. Such enhancer comprises any material which aids the surfactant in solubilization. For example, bile salts are particularly effective such as sodium cholate, sodium deoxycholate, etc.

In another preferred embodiment, the surfactant is of the above formula is which R is alkyl, e.g. the surfactant is lauric acid diethanolamide or oleic acid diethanolamide.

The enzyme is derived from an animal source, e.g. animal pancreatic tissue, or a microbial source.

Biological fluid samples which can be treated by the method of the invention include human serum and plasma, for example.

When in the formula given above, x + y is greater than 2, a preferred reagent is one in which x + y is 5, R is alkyl, preferably lauryl, and the enzyme is a lipase. Formulations with this reagent preferably include polyethylene glycol p-isooctyl phenyl ether or other suitable surfactants.

Another preferred reagent which will produce effective clearing of turbid samples in the pH range of 2 to 10 is one which contains a mixture of two surfactants, namely, lauric acid diethanolamide (x = y = 1) and epoxylated lauric acid (x + y = 5).

Among the surfactants which may be used are lauric acid diethanolamide, myristic acid diethanol-amide, capric acid diethanolamide, oleic acid diethanolamide and coco acid diethanolamide.

When a reagent of the invention is combined with a turbid biological fluid sample, the turbidity is reduced or cleared the sample can then be accurately photometrically assayed or analyzed. Typical assays that can be carried out utilizing the reagents of the invention include cholesterol, triglycerides and creatine phosphate kinase determinations.

The method and reagent of this invention permit the determination of components in biological fluid samples in a clear free state substantially without turbidity interferences. In addition, because of the interaction between the particular surfactant and enzyme employed, they permit the use of lesser amounts of enzyme than are normally used as in the case of cholesterol determination. In this latter regard, the employment of smaller amounts without lessening in reates of reaction can be viewed as an improvement in the rate of enzymatic reaction.

The most common clinical determination of cholesterol in a biological fluid is the total cholesterol which includes both free cholesterol and cholesterol esters. Both cholesterol and its esters are present in serum with other lipids and various proteins in micromolecular complexes called lipoproteins and cholesterol esters normally exist as a major component (60-80%) of the total cholestrol. They are generally water insoluble and are normally buried inside the complex and inaccessible to enzymes. In the determination of total cholesterol by a wholly enzymic method, whether automated or manual, both cholesterol and cholesterol esters must first by liberated by a suitable surfactant. Cholesterol esters are then hydrolyzed by cholesterol esterase to yield free cholesterol which is, in turn, oxidized by cholesterol oxidase to form cholestenone and hydrogen peroxide.

The herein disclosed method can be applied in automated fashion as by the employment of an automatic analyzer, or it can be done manually.

In preparing the formulations for use in assaying by the method of this invention, an aqueous solution is formulated which may contain, in addition to the surfactant and enzyme, other reference materials which are known in the art and are utilized for such purpose.

For example, in cholesterol assaying the following components are normally employed in the ranges shown herein below:

Component	*Cholesterol Assay*
Peroxidase	0.8 - 2.0 U/l
Cholesterol oxidase	0.025 - 0.3 U/ml
Cholesterol esterase	.025 - .3 U/ml
Surfactant	.05 - .5 g/dl
Sodium cholate	0.05 - 0.5 g/dl
Sodium p-Hydroxybenzoate	2.5 - 6 g/dl
4-Aminoantipyrine	0.5 - 2.0 mM
Maleic acid	0.1 - 0.5 M
pH	5.5 - 7.0
Sample/Reagent ratio	100 - 400

In the above formulation, it is preferred to use cholesterol esterase from an animal source, e.g. pancreas; however, equivalent results are obtained with cholesterol esterase from a microbial source.

EXAMPLE I

Clearing as a function of cholesterol esterase

Three ml of clearing reagent which contains potassium-maleate (0.1 M), sodium cholate (0.25 g/dl), lauric acid diethanolamide (0.2 g/dl, cholesterol esterase (0.08 U/ml to 0.8 U/ml), final pH 6.0, is mixed with 0.025 ml of lipemic serum (triglycerides or about 1400 mg/dl). The reaction mixture turns clear, within 10 minutes at 45°C. The cholesterol esterase for clearing can be obtained from pancreas or from microorganisms.

EXAMPLE II

Use for cholesterol determination

For end point chemistry, as illustrated in this Example, the ingredients for cholesterol assay are included in

the clearing reagent.

Three ml of a formulation which contains cholesterol esterase (0.125 U/ml), cholesterol oxidase (0.125 U/ml), peroxidase (1.6 U/ml), 4-aminoantipyrine (0.6 mM), sodium hydroxybenzoate (25 mM), sodium cholate (0.25 g/dl), lauric acid diethanolamide (0.2 g/dl) and potassium maleate (0.1 M), pH 6.0, is mixed with 0.025 ml of lipemic serium sample. The mixture is then incubated at 45°C for 4-5 minutes. The total cholesterol is then determined by measuring the colour intensity at 520 nm. Without the clearing effect of lauric acid diethanolamide and cholesterol esterase, the determination of cholesterol in turbid samples always gives erroneous results.

EXAMPLE III

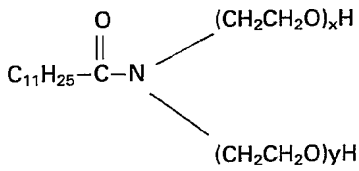
Clearing by candida lipase (candida cylindracea)

Three ml of a clearing reagent containing lauric acid diethanolamide (0.2 g/dl), Na cholate (0.25 g/dl) and lipase (25 U/ml) and maleate buffer (0.1 M), pH 6.0, are mixed with 0.025 ml lipemic serum. The turbid sample will turn clear after about 5 minutes incubation at 45°C.

When an equivalent amount of clearing agent comprising a mixture of lauric acid diethanolamide and epoxylated lauric acid ($x + y = 5$) is used, comparable clearing results.

EXAMPLE IV

Three ml of a clearing reagent containing a surfactant of the formula:



in which $x + y = 5$ (0.2 g/dl), Triton X-100 (0.4 g/dl), potassium maleate (0.2 M) and lipase (25 U/ml), pH 6.0, is mixed with 0.05 ml lipemic sample and incubated at 45°C. The turbid sample turns clear after 3 minutes.

The rate of clearing is enchanced by increasing the buffer concentration.

Similar results are obtained by using higher concentrations of epoxylated lauric acid ($x + y = 5$) without the assistance of Triton X-100.

EXAMPLE V

A. *Formulation*

A diagnostic reagent formulation is prepared as a one liter aqueous solution using the following ingredients.

Ingredient	*Concentration*
Malic Acid	11.6 g
KOH	10.0 g
EDTA (K_2)	2.7 mM
Na Cholate	5.8 mM
Na p-Hydroxybenzoate	25.0
4-Aminoantipyrine	0.6 mM
Lauric Acid Diethanolamide	2.0 g
Cholesterol Esterase	125 Units
Cholesterol Oxidase	125 Units
Horseradish Peroxidase	800 Units

The pH is adjusted to 6.0.

The reagent system may be stored and used in the form of an aqueous solution or the solution may be freeze-dried by conventional means and reconstituted with water when ready for use.

B. *Assay - total cholesterol determination*

Three ml of the above reagent is mixed with 0.025 ml of serum or reconstituted serum standard which contains up to 500 mg/dl cholesterol. The reaction is carried out at 45°C for 4 to 5 minutes. The absorbance of samples at 525 nm is measured against the reagent blank.

EXAMPLE VI

Clearing in determination of creatine phosphate kinase (CPK) activity of lipemic serum

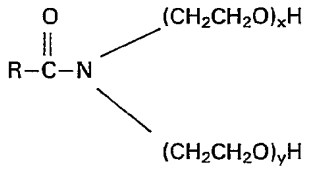
Two ml of imidazole-acetate buffer, 0.1 M, pH 6.7, containing lauric acid diethanolamide (0.4%), pancreatic cholesterol esterase (25 U/dl), Na cholate (0.25 g%) and thiolglycerol (20 mM), is mixed with 0.05 ml of

lipemic serum. After 15 minutes' incubation at 37°C, the turbidity at 340 nm drops from 2.3 O.D. to 0.02 O.D. The clear sample is then mixed with 1 ml of CPK reagent which contains creatine phosphate (116.7 m) mM), ADP (6.7 mM), AMP (16.7 mM), EDTA (6.7 mM), NADP (6.7 mM), hexokinase (125 U/dl), glucose 6-phosphate dehydrogenase, G6PDH (100U/dl) prepared in 0.1 M imidazole-acetate buffer, pH 6.7. The activity of CPK is monitored at 340 nm and 37°C as the conventional method.

It should be understood by those skilled in the art that various modifications may be made in the present invention without departing from the spirit and scope thereof as described in the specification and defined in the appended claims.

CLAIMS

1. A reagent for reducing turbidity in a biological fluid sample, which reagent comprises at least one surfactant of the formula:



wherein R is alkyl or alkenyl containing from 5 to 17 carbon atoms; x and y are integers whose sum is no greater than 11; and an enzyme selected from cholesterol esterase or lipase or mixtures thereof.

2. A reagent according to claim 1, which is in aqueous buffered form, and wherein the surfactant comprises from about 0.05 g/dl to about 2.5 g/dl; and wherein the enzyme is cholesterol esterase and comprises at least about 0.025 U/ml of the aqueous buffered reagent; the aqueous buffered reagent having a pH of from about 5.5 to about 8.0.

3. A reagent according to claim 2, wherein the surfactant comprises from about 0.1 to about 0.5 g/dl.

4. A reagent according to claim 2 or 3, wherein x + y is no greater than 5.

5. A reagent according to claim 2, 3 or 4, wherein the buffer is a maleate which is present in a concentration of from about 0.05 M to about 0.5 M, and the pH of the aqueous buffered reagent is from about 5.0 to about 7.0.

6. A reagent according to claim 1, which is in aqueous buffered form and in which said surfactant comprises from about 0.05 g/dl to about 2.5 g/dl, and wherein said enzyme, which is a lipase comprises at least about 1.0 U/ml of the aqueous buffered reagent, the pH being from about 2.0 to about 10.0.

7. A reagent according to claim 6, wherein the surfactant comprises from about 0.1 to about 0.5 g/dl and the pH is from about 5.5 to about 8.0.

8. A reagent according to claim 6, wherein the buffer is maleate, citrate, succinate, Tris or borate buffer, in a concentration of from about 0.05 M to about 0.5 M.

9. A reagent according to any preceding claim, wherein said surfactant is of formula in which x and y are each 1.

10. A reagent according to claim 9, in which R is alkyl.

11. A reagent according to claim 10, wherein said surfactant is lauric acid diethanolamide, myristic acid diethanolamide or capric acid diethanolamide.

12. A reagent according to claim 9, wherein R is alkenyl.

13. A reagent according to claim 12, wherein said surfactant is oleic acid diethanolamide or coco acid diethanolamide.

14. A reagent according to any preceding claim, in which said enzyme is derived from an animal or microbial source.

15. A reagent according to claim 1, 2, 3, 4 or 5, in which ssid enzyme is cholesterol esterase derived from animal pancreatic tissue.

16. A reagent according to any preceding claim, which also includes a solubility enhancer.

17. A reagent according to claim 16, in which said solubility enhancer is sodium cholate or sodium deoxycholate in an amount to provide about 0.25 g/dl of the reagent.

18. A reagent according to claim 1, wherein said surfactant is of the formula in which R is alkyl and the sum of x and y is 5, and said enzyme is a lipase.

19. A reagent according to claim 18, which includes polyethylene glycol p-isooctyl phenyl ether (Triton X-100).

20. A reagent according to claim 1, in aqueous buffered form, in which said surfactant is a mixture of lauric acid diethanolamide and epoxylated lauric acid (x + y = 5), said mixture comprising from about 0.05 g/dl to about 2.0 g/dl of the aqueous buffered reagent, the pH being from about 2.0 to about 10.0.

21. A reagent according to claim 1 substantially as herein described in the Examples.

22. A method of reducing the turbidity in a biological fluid sample to be photometrically assayed, which comprises combining said sample with a reagent as claimed in any of claims 1 to 21.

23. A method according to claim 22, in which said biological fluid sample is a human serum sample.

24. A method according to claim 22 in which said sample is photometrically assayed for cholesterol, the said reagent comprising cholesterol esterase.

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